CHARACTERIZATION OF QUORUM SENSING MEDIATED BIOFILM FORMATION AND ITS INHIBITION IN *Cronobacter sakazakii* STRAINS

SYNOPSIS

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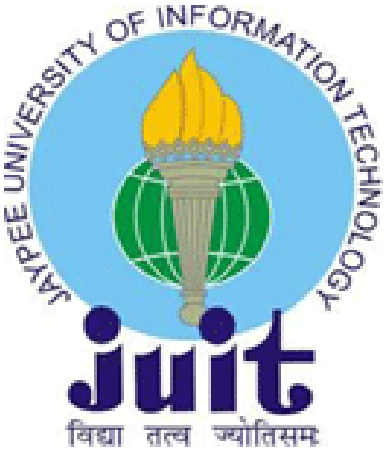

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INTRODUCTION

Cronobacter species, more specifically *C. sakazakii* is an emerging Gram-negative pathogen associated with severe neonatal systemic infections predominantly in pre-term or low birth weight infants and thus has attracted the attention of public health authorities and researchers in the past as reviewed by Singh et al. (2015). The genus *Cronobacter* consists of seven species: *Cronobacter dublinensis, Cronobacter malonaticus, Cronobacter muytjensii, Cronobacter sakazakii, Cronobacter turicensis, Cronobacter universalis and Cronobacter condimenti* (Joseph et al., 2012). Among these, *C. sakazakii*, *C. turicensis* and *C. malonaticus* are reported to cause neonatal infection. Although the incidence of disease is low, the fatality rate has been reported to be up to 80% and and survivors often had severe neurological and developmental disorders (Kalyantanda *et al*. 2015). The International Commission on Microbiological Specification for Foods (ICMSF) has classified *C. sakazakii* as a 'severe hazard for restricted populations, life-threatening or with substantial chronic sequelae over long duration'. Furthermore, the World Health Organization (WHO) and Food and Agriculture Organization (FAO) in 2008 issued a joint call to the scientific community for attaining more data on this organism (WHO/FAO). In the Indian context, there has not been a far-reaching report on association of this pathogen with the different food commodities. However, a clinical case study was reported by Ray et al. (2007) which described two cases of *C. sakazakii* infections; one preterm very low birth weight neonate with meningitis and a two month infant with bacteraemia for the first time in India. The another report was a case of urinary tract infection due to C. *sakazakii* in a 63-year-old lady. Despite treatment, she expired six days after hospitalization (Bhat et al. 2009).

The bacterium is ubiquitous and has been isolated from a wide spectrum of environmental sources including water, soil, dust from households and food production lines, as well as from foods such as fruits, vegetables, herbs, cereals and grains. Contamination in infant food, especially in powdered infant formula (PIF), has made this pathogen a great threat to infants due to their immature immunity and intake of PIF as their main food (Jaradat et al. 2014)

Realizing the fatality associated with the *C. sakazakii*, recent research studies have focused on the mechanisms of infection and the virulence factors of this pathogen. The *in vitro* studies using human-derived cell lines and *in vivo* studies using suckling mice or rats have shown that *Cronobacter* can adhere to and invade human intestinal cells, invade the blood/brain barrier, and replicate in macrophages (Nair and Venkitanarayanan 2007; Singamsetty et al. 2008; Nair et al. 2009). However, little is known about the pathogenesis of *Cronobacter* at the molecular level. As the major virulence marker of its pathogenicity, Outer membrane

protein (Omp), is one of the best characterized virulence factors and plays an important role in the binding and invasion of endothelial cells (Kim et al. 2010). After the colonization and invasion of host tissues or cells, *Cronobacter* must exploit some other mechanism to avoid the immune system or survive within the host cells. Nevertheless, this mechanism remains incompletely understood. The expression of outer membrane protease Cpa (*Cronobacter* plasminogen activator) is reported to increase after the entry of *Cronobacter* in the host (Franco et al. 2011). The genome of *C. sakazakii* BAA-894 illustrated that this strain possesses two plasmids, pESA2 (31 kb) and pESA3 (131kb) (Kucerova *et al*., 2010). pESA3 encodes an outer membrane protease (named *Cronobacter* plasminogen activator) that was shown to provide serum resistance to *C. sakazakii* and may enhance its spread and invasion in a host. However, most of the genes identified require further experimental verification. Although many efforts have been made to explore the pathogenesis of *Cronobacter*, in comparison with other microbial pathogens, knowledge is limited for this pathogen. Very few studies was done in India on virulence mechanism of *C. sakazakii*. Sivamaruthi et al (2011; 2015a; 2015b) described the physiological changes and an induction of host immunity mediated antimicrobial genes by *C. sakazakii* ATCC BAA-894 in Caenorhabditis elegans taken as a model.

Epidemiological investigation of outbreaks of *Cronobacter* spp. contagions indicated contaminated reconstituted PIF as a source of contamination, when these microorganisms were isolated from both reconstituted PIF as well as from milk feeding equipment and utensils. The latter may be due to its ability to adhere and form biofilms on many surfaces, including silicone, latex, polycarbonate (used in the feeding bottle manufacture) and stainless steel (Lehner et al. 2005). The biofilm, generally defined as a structured community of bacterial cells, enclosed in a self-producing matrix and adherent to an inert or living surface (Costerton et al.1999) is reported to play a major role in survival of *C. sakazakii* under stress conditions. The ability of bacteria to attach to and form biofilms on surfaces may influence their persistence during manufacturing and retail storage of food products, their ability to contact food, and colonize individuals to cause disease (Kumar and Anand, 1998). Bacterial growth as a biofilm community offers many advantages over single-cell growth, including survive the stress conditions, increase resistance to antimicrobial factors such as antibiotics, disinfectants, and sanitizers. Various factors such as flagella, outer membrane proteins and extracellular polymeric substances (EPS) are involved in biofilm formation (Jaradat et al. 2014) The EPS, has been shown to play a substantial part in determining the architecture of a

biofilm among the members of the Enterobacteriaceae. Sharma and Prakash (2013) observed the differential protein pattern in 9 isolates of *C. sakazakii* cultivated in biofilm versus agar-surface-associated and planktonic cells were observed. But authors characterized the isolates using the 16S rRNA sequencing only and according to recent study, this was not sufficient to discriminate between all *Cronobacter* spp (Forsythe et al. 2015).

Bacterial processes involved in biofilm formation and virulence, are often controlled by quorum sensing (QS), a mechanism based on the production, release and detection of signaling molecules of low molar mass. Extracellular concentrations of signal molecules are sensed by the bacteria and, upon reaching a population density-dependent threshold, they are detected by the cells, which in turn induce target gene expression in a coordinated fashion (Waters & Bassler 2005) To date many structurally unrelated signal molecules have been identified, including *N*-acyl-homoserine lactones (AHLs) in Gram-negative bacteria, oligopeptides in many Gram-positive bacteria and autoinducer-2 (AI-2), which are thought to serve as a signal for interspecies communication (Ryan et al. 2008). These QS regulatory mechanisms have also being suggested as a novel target for developing advanced strategies to prevent infections (Rasmussen & Givskov 2006). Although, several natural and synthetic anti-QS compounds have been reported where extracts of various natural products (Rasmussen et al. 2005) and essential oils of several plants (Szabo et al. 2010) have shown anti-QS effects. Earlier reports on anti-biofilm ability of trans-cinnamaldehyde from cinnamon indicated that the compound at sub-inhibitory concentration of TC is able to inhibit biofilm synthesis and inactivate mature biofilms of *C. sakazakii* on different abiotic substrates; however no investigation was conducted on QS mechanisms.

As the *Cronobacter* spp. is a newly classified genus and needs more investigations for better understanding this unique group of organism. As a virulent species, it causes high mortalities in the neonates; therefore, it is important to highlight the presence and to understand which gene products are responsible for the pathogenicity of the bacteria. Further, the present study also investigates the biofilm formation, its composition and identification of QS molecules in *C. sakazakii*. The study also investigates the quorum quenching activities of the plant extracts in *C. sakazakii* and their role in inhibition of biofilm formation.

OBJECTIVES

- **Isolation and characterization of *C. sakazakii* from different sources and detection of selected virulence traits in the *C. sakazakii.***
- **Characterization of biofilm in *C. sakazakii* and determination of key molecules involved in quorum sensing.**
- **Evaluation of quorum sensing inhibitory activity of plant extracts against biofilm forming *C. sakazakii.***

MATERIAL AND METHODS

Objective 1: Isolation and characterization of *C. sakazakii* from different sources and detection of selected virulence traits in the *C. sakazakii.*

Collection of samples

A total of 219 samples of different food products (raw and processed): Powdered Infant Formulae (Nestle and Amul), infant food formula (Nestle), medicinal plants, herbs, and spices were purchased from local outlets along with environmental samples (soil, water) were tested for the presence of *Cronobacter* spp. The standard strains of *C. sakazakii* ATCC 12868 and E604 (kindly gifted by Dr. Ben Davies Tall, FDA, USA) were used for the comparison.

Enrichment and isolation

A 25 g of each sample collected in sterile vials was added to 225 ml buffered peptone water (BPW) and mixed well for pre-enrichment (ISO 22964:2006). Following an overnight incubation at 37°C, 10 ml of the pre-enrichment culture was inoculated into 90 ml of Enterobacteriacae Enrichment (EE) broth and incubated overnight at 37°C. A 10 µl volume of the selective enrichment was then streaked onto a tryptone soya agar (TSA). All colonies that appeared on TSA were picked and subjected to further characterization using chromogenic media *Enterobacter sakazakii* agar (Himedia laboratories, India). The presumptive isolates were confirmed by biochemical tests.

Biochemical differentiation of *Cronobacter* species

The isolates were identified by biochemical tests for the differentiation of *Cronobacter* spp., as per the tests defined for members of Enterobacteriace including Indole production, Methyl red, Voges-Proskauer, Citrate utilisation test as per the Standard Microbiological protocol.

Determination of carbohydrate fermentation was also done in carbohydrate basal medium with phenol red (Ewing, 1986) as pH indicator. The carbohydrates tested were inositol, sorbitol, X-methyl-D-glucoside, lactose, fructose, dextrose, xylose and sucrose. All the results were compared with the standard strains.

DNA extraction and Plasmid isolation

An aliquot of 2 ml of each bacterial culture was used for DNA extraction using phenol:chloroform:iso-amyl alcohol method as described by Sambrook et al. [33]. In parallel, plasmids were isolated from a 3 ml aliquot from each broth culture using Qiaprep spin miniprep kit as per the instructions by manufacturer. The detection of plasmids was confirmed using the repA targeted PCR assay as described by Franco et al. [11]

16S rRNA target PCR

The isolates were confirmed by genus-specific and species-specific PCR as the primer sequences described in Table 1. The conditions for PCR amplification targeting16S rRNA gene and ITS region were according to Jaradat et al. [2009]. The PCR products were then analyzed in 1.5% (w/v) ethidium bromide-stained agarose gel. A non-template control was included in each run.

rpoB amplification of *C. sakazakii* strains

The isolates were analysed for RNA polymerase beta-subunit encoding gene (rpoB) amplification as an alternative tool for universal bacterial genotypic identification. The rpoB analysis confirmed 38 *C. sakazakii* isolates which were investigated for the further study.

Multilocus sequence typing (MLST) of C. sakazakii strains

The isolates were further assigned for MLST analysis, targeting elongation factor (*fus*A). The MLST analysis confirmed five *C. sakazakii* isolates using MLST database (http://www.pubMLST.org/cronobacter) which were investigated for the further study.

Virulence determinants through biochemical assays

Various biochemical assays for determination of virulence traits were conducted as per standard protocols defined for *Cronobacter* sp.

- For inositol fermentation test, the overnight grown culture of *C. sakazakii* isolates in Tryptone Soy Broth (TSB) was centrifuged and the cell pellet was suspended in a carbohydrate basal medium (Peptone 10%; Sodium chloride 5%; Phenol red 0.018%) with inositol (1mg/ml) and phenol red as indicator. The culture was incubated for for 24 h at 37°C. A positive response was shown by a change in color from reddish to yellowish.

- The DNase test was performed by culturing the isolates on DNase agar for 24 h at 37°C and pouring 2 ml of 1 N HCl after incubation (Smith et al. 1969). Only strong DNase activities displaying clear zone around growth were taken as positive.
- The ability of isolates for production of hemolysin was determined by streaking overnight growth culture on sheep blood agar plates (Himedia Laboratories, India) and incubated at 37^{o}C for 24 h (Keller et al. 1988). Hemolysis was recorded as α-hemolysis, β-hemolysis, and negative (no hemolysis).
- The siderophore production was determined using the chrome azurol S (CAS) agar (Himedia Laboratories, India) well diffusion assay (Shin et al. 2001).
- The chitinaase activity was determined as zone of hydrolysis on colloidal chitin agar by inoculating the overnight grown culture of isolates on the colloidal chitin agar medium with incubation at 37^{o}C for 96 h (Kaur et al. 2012).
- The sialic acid utilization was determined by inoculation an overnight *C. sakazakii* cultures in M9 minimal media supplemented with 1 mg/ml of *N*-acetylneuraminic acid (Sialic acid) (Joseph et al. 2013). The tubes were incubated at 37^{o}C for 24 h and the growth of each isolate was monitored by measuring the absorbance at 595 nm.

Detection of virulence associated genes by PCR

The PCR was conducted in individual reactions using primers for the following nine genes: *omp* (outer membrane protein), *hly* (hemolysin), *cpa* (*Cronobacter* plasminogen activator), *chiF* (chitinaseF), *eitA* (iron acquisition gene), BAM (zinc containing metalloprotease), *sip* (siderophore), *fhaB* (filamentous hemagglutinin) and *iucC* (iron acquisition gene) as per the primers and conditions described in literature (Jaradat et al. 2009; Franco et al. 2011; Cruz et al. 2011; Yan et al. 2011) (Table 1). The amplified products were analyzed in 1.5% agarose gel along with 1 kb DNA marker (Gene Ruler–Fermentas) by staining with ethidium bromide.

Objective 2: Characterization of biofilm in *C. sakazakii* and determination of key molecules involved in quorum sensing.

Screening and Quantification of Biofilm Formation

The screening and quantification of biofilms among different isolates was determined using standard crystal violet microtiter plates assay as described by Boddey et al. (2006). TSB medium was used as the growth medium and cultures were incubated at 37°C for 48 h. After crystal violet (CV) staining, the absorbance at 570 nm was measured using 540 nm using Go

scan microplate reader (Thermo, USA). The extent of biofilm formation was measured as depicted by Naves et al. (2008) : (i) BF=AB-CW, where BF is the biofilm formation, AB is the OD_{540nm} of stained attached bacteria and CW is the OD_{540nm} of stained control wells containing bacteria-free medium only; (ii) BF=AB/CW; and (iii) SBF= (AB- CW)/G in which SBF is the Specific Biofilm Formation index and G is the OD_{600nm} of cells growth in suspended culture. The SBF index in *C. sakazakii* isolates was classified semi-quantitatively in three categories on the basis of absorbance for each of the formula used: strong (>2.5), moderate (1.5-2.5) and weak (<1.5).

Detection of genes involved in biofilm formation

The DNA was extracted from overnight grown culture using phenol:chloroform:iso-amyl alcohol method. The primers and PCR parameters for detection of genes (*bcsC, bcsG, bscA, flgJ, fliD* and *flhE*) involved in biofilm formation were reported in Ye et al. (2015) (Table 1).

Table 1: Details of primer pairs and PCR running conditions used in this study.

S.No	Primer	Sequence 5' to 3'	Region	Amplicon	PCR running conditions
For Molecular Identification					
1.	Saka 1a Saka 1b	ACAGGGAGCAGCTTGCTGC TCCCGCATCTCTGCAGGA	V1 V3	952 bp	95°C for 4 min; 30 cycles of 95°C for 60 sec each; 50°C for 1 min; 72°C for 90 sec; 4 min at 72°C for 4 min.
2.	SG-F SG-R	GGGTTGTCTGCGAAAGCGAA GTCTTCGTGCTGCGAGTTTG	ITS-G	282 bp	94°C for 10 min; 30 cycles of 94°C for 30 sec each; 57°C for 1 min; 72°C for 1 min; 72°C for 5 min.
3.	CsakF CsakR	ACGCCAAGCCTATCTCCGCG ACGGTTGGCGTCATCGTG	rpoB	514 bp	96°C for 1 min; 30 cycles of 96°C for 1 min, 58°C for 1 min, 72°C for 2 min, 72°C for 5 min.
For Detection of Plasmids					
4.	InF2F InF2R	GAGACTGGCAGACCCGGCATA TGCGTTCACGAGCTGCCTGG	*repA*	398 bp	94°C for 3 min, 30 cycles 59°C for 30 s/72°C for 40 s, 72°C for 5 min
5.	H1F H1R	CGCCTCAGTGTGTTCACTCCG TAGCTCCCATAGACTCGGATC	*repA*	551 bp	94°C for 3 min, 30 cycles 52°C for 30 s/70°C for 60 s72°C for 5 min
6.	F1B2F F1BR	GATGCGCCTTGGCCTGTTTGT GCACAGCTTCACGAACTCCAC	IncF1B	264 bp	94°C for 3 min, 30 cycles 56°C for 30 s/72°C for 30 s72°C for 5 min
For Virulence traits					
7.	hly F hlyR	CTAGGGTAACGGACTGTCACAGAT CTAGGAAGAAGCGTAAGCGTCTGA	ESA_00432 409290–410170	880 bp	94 °C for 3 min; 30 cycles at 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 1 min; 72 °C for 10 min
8.	ESSF ESSR	GGATTTAACCGTGAACTTTTCC CGCCAGCGATGTTAGAAGA	*ompA*	469 bp	94 °C for 2 min; 30 cycles 94°C for 15 s each; 58°C for 15 s; 72°C for 30 s; 72°C min for 5 min
9.	cpaF cpaR	GACAACCCTGAGTTCTGGTAAC ATGCGTATTTCTGCTGGTAA	*cpa*	306 bp	94 °C for 2 min; 30 cycles 94°C for 30 s each; 56°C for 30 s; 72°C for 30 s; 72°C min for 5 min
10.	sak_Chi_F sak_Chi_R	ATGGCTACMAGYAAAYTRATYCAGGG CACCTGRTAGTTRTGVCCTTTCCAGC	*chi*	2.2 kb	95 °Cfor 2 min; 30 cycles at 95 °C for 10 s, 55 °C for 30 s, and 72 °C for 2 min; 72 °C for 10 min
11.	EitAF EitAR	CCTTTTTCACGGCGTCGAGCTG TCTCTTCTGGTTCTCCAGCGCG	*eitA*	280 bp	94 °C for 3; 30 cycles 94°C for 60 sec each; 60°C for 30 s; 72°C for 30 s; 72°C min for 5 min
12.	BAM122 BAM123	AWATCTATGACGCGCAGAACCG AAAATAGATAAGCCCGGCTTCG	*zpx*	350 bp	Hot start PCR: activated by incubation for 15 min at 95°C; 30 cycles 95°C for 60 s each; 51°C for 60 s; 72°C for 60 s; 72°C min for 7 min
13.	sip F sip R	CTAGGCAAAAGAATCGACAAAGGG CTAGGTTGTTGTCTTTATCCGTTC	ESA_03485 c3431208– 3432142	934 bp	94 °C for 3 min; 30 cycles at 94 °C for 30 s, 57 °C, for 30 s, and 72 °C for 1 min; 72 °C for 10 min
14.	fhaF fhaR	GTGGCGAACCCGTATGGCATTAC TGTCGCCTGCTATCTGCGCGTTG	*fhaB*	804 bp	94 °C for 3 min; 30 cycles 94°C for 15 sec each; 60°C for 15 s; 72°C for 30 s; 72°C min for 5 min
15.	IucCF	TGCAGTGCCTGATGTCAGGCCAT	*iucC*	660 bp	94 °C for 2; 30 cycles 94°C for 15 sec each; 60°C for 15 s; 72°C

	IucCR	ACGCCAAACATCTCCTGATAGCG			for 30 s; 72°C min for 5 min
For Biofilm characterization					
16.	BCSA-F BCSA-R	AAGAAGAGTACGTGGACTGGGTGA CGCCGAGGATAATCAGGTTGTAG	-	171 bp	94^{o}C/3min, 30 cycles 94^{o}C/2min, 59.5^{o}C/30 sec,72^{o}C /45sec,72^{o}C /5min
17.	BCSG-F BCSG-R	GACGGGCTATCTGAATTTCCAC GCCAGGTATCATGCCAGAACA	-	146 bp	94^{o}C/3min, 30 cycles 94^{o}C/2min 58.7^{o}C/30 sec,72^{o}C /45sec,72^{o}C /5min
18.	BCSC-F BCSC-R	AGATTTGAGCGGTTATTCTTTAGGC TCGGTCTTCGTGCGGAGTG	-	156 bp	94^{o}C/3min, 30 cycles 94^{o}C/2min 58.7^{o}C/30 sec,72^{o}C /45sec,72^{o}C /5min
19.	FLGJ-F FLGJ-R	TCAGGTGCCGATGAAGTTTG GCCCTTTCCAGGACGATGT	-	312 bp	94^{o}C/3min, 30 cycles 94^{o}C/2min 58.7^{o}C/40 sec,72^{o}C /45sec,72^{o}C /5min
20.	FLHE-F FLHE-R	CATTACTGACGCTGCCTGTCC GTAGTGCCCGTCTGGTCTTCC	-	256 bp	94^{o}C/3min, 30 cycles 94^{o}C/2min 58.2^{o}C/30 sec,72^{o}C /45sec,72^{o}C /5min
21.	FILD-F FILD-R	ATCGAGATCGAGCGTTCCAC CGCCCTTATCAACTTTGACGTATT	-	211 bp	94^{o}C/3min, 30 cycles 94^{o}C/2min 58.7^{o}C/30 sec,72^{o}C /45sec,72^{o}C /5min

Extraction of Extracellular Polyscaccharide (EPS) and its characterization

The EPS was extracted by the modified procedure of Onbasli and Aslim (2009). Five ml of overnight culture was centrifuged at 15,000g for 5 min and boiled for 15 min at 100 ^{o}C. A 100 ml of trichloroacetic solution was added to the suspension and incubated for 1 h at 37^{o}C. The mixture was kept in ice water for 30 min and centrifuged at 15,000 g for 20 min. The supernatant containing EPS was pooled with equal volume of ethanol. The mixture was kept at -20^{o}C for 1 h and then centrifuged at 15,000g for 20 min again. The precipitate was washed using 95% ethanol and the final precipitate was dissolved in 1 ml of deionized distilled water and was evaluated for the total carbohydrate content and protein using Phenol-Sulfuric Acid (PSA) method and Bradford method. The Fourier transform infrared (FTIR) (Agilent Cary 630 FTIR system) spectroscopy was done using KBr as the reference.

Detection of Quorum sensing mechanisms

Based on the SBF index, isolate N13, N15 and N112 were selected for further work.

Screening and characterization of AHL

The AHL production among the isolates was screened using well diffusion and test tube assay described in Mukherji and Prabhune (2015). For the well diffusion assay, the test cultures were added in wells of the LB agar plate overlaid with CV026. The testing for AHL production against *A. tumefaciens*NTL4(pZLR4) was done in a similar way supplementing the agar with50 mg/ml X-gal. The production of violacein and blue halo around colony was taken as an AHL-positive response of CV026 and *A. tumefaciens* NTL4(pZLR4).

For test tube assay, a 500 µl of overnight broth culture of *A. tumefaciens*NTL4(pZLR4) was added to 10 ml of NB medium along with 1ml inoculum of *C. sakazakii* strains and kept for overnight at 28 ^{o}C. A 2 ml of culture medium was withdrawn and centrifuged at 15,000 g for 10 min. The cell pellet was solubilised in 2 ml of dimethyl sulfoxide (DMSO), and centrifuged and the development of a blue-green color indicating the presence of acyl-AHL was measured at 630 nm.

Extraction of AHL

The extraction of AHLs was done as described by Shaw et al (1997). The isolates were cultured in 500 ml of TSB for 18 h at 37 ^{o}C and 200 rpm. The supernatant of early stationary-phase culture was extracted twice with equal volumes of ethyl acetate.The organic phases were combined and dried by rotary evaporation. The residue was resuspended in 500 µl of DMSO and further characterized for FTIR, HPLC and mass spectrometry analysis.

The LC-HRMS analysis of extracted AHL in acetonitrile was performed on Thermo Scientific, Hybrid Quadrapole Q-Exactive orbitrap mass spectrometer. The chromatographic

separation was carried out using LC (Accela 1250 pump), Thermo Scientific Hypersil ODS C18 column (Fekete et al. 2007). The isocratic solvent system of acetonitrile and water was used in the ratio 99:1 holding for 5 min. The type of AHL was detected based on the m/z (mass to charge ratio).

Objective 3: Evaluation of quorum sensing inhibitory activity of plant extracts against biofilm forming *C. sakazakii.*

Plant products and extract preparation

The nine plant products (*Piper nigrum, Coriandrum sativum, Cuminum cyminum, Syzygium aromaticum,* Trigonella foenum-graecum, *Myristica fragrans, Zingiber officinale, Allium sativum and* Cinnamomum verum) were purchased from the local outlets and the extractions were made by following the method of Choo et al. (2006), with slight modifications. Each of the plant products was washed in sterile water, dried, and powdered using a mixer grinder. For the preparation of methanolic extract, powdered samples (50 g) were soaked in 300 mL of methanol for overnight under shaking at 100 rpm at 30 °C. The methanol phase was collected and concentrated by rotary evaporator to obtain the dried residue. The residues were re-dissolved with 10% dimethyl sulfoxide (DMSO) and stored at -20 °C until further use.

Anti-quorum sensing activity of plant extracts

The anti-quorum sensing activity of the extracts was evaluated using indicator strains with well diffusion as well as test tube assay.

Well diffusion assay

The methanolic plant extracts were screened for anti-QS activity using an AHL-based *in vitro* QS assay using two biosensor strains, *C. violaceum* CV026 and *A. tumefaciens* NTL4(pZLR4) by the agar-well diffusion test. A 50 µL of overnight broth culture of *C. violaceum* CV026 (10^5 cfu/mL) was added to 10 mL of LB soft agar along with 1.25 µM of C6-AHL (99%, Cayman Chemicals) as QS standard molecule and the mixture was overlaid onto LB agar plates. After solidification of overlay layer, wells of 6 mm diameter were dug using a sterile corkborer. The methanolic plant extract (50 µL) was added to respective wells and the plates were incubated overnight at 28 ^{o}C in upright position (McLean et al. 2004; Mukherji and Prabhune 2015). The testing for QS inhibition against *A. tumefaciens* NTL4(pZLR4) was done in a similar way supplementing the agar with 50 mg/mL X-gal along with 1.25 µM of 3-oxo-C8 AHL (99%, Cayman Chemicals) as standard AHL. The inhibition of QS was detected by the presence of colorless zone with viable cells around the well.

Test tube assay

The qualitative effect of extracts on the QS-controlled production in *C. violaceum* CV026 and *A. tumefaciens* NTL4(pZLR4) was determined as described previously with some modification (Choo et al. 2006). Briefly, 10 mL LB and NB medium containing 50 µL of each plant extracts was inoculated with *C. violaceum* CV026 and *A. tumefaciens* NTL4(pZLR4) along with 1.25 µM of C6 AHL and 3-oxo-C8 AHL, respectively. Then each tube were incubated for 24 h at 28 ^{o}C in an orbital shaking incubator (130 rpm). Violacein was extracted in DMSO from the cells and was quantified spectrophotometrically at optical density 580 nm in a UV-Vis spectrophotometer. Simultaneously, absorbance of the supernatant containing blue-green colouration was measured at a wavelength of 630 nm using DMSO as the blank. The percentage of inhibition was calculated by following the formula: percentage of inhibition = (control $_{OD}$ − test $_{OD}$ / control $_{OD}$) × 100.

Inhibition of *C. sakazakii* biofilm formation

The effect of extracts on the biofilm formation of *C. sakazakii* isolates was determined by quantifying the biofilm biomass through microtiter plate (MTP) assay (Thenmozhi et al., 2009). In brief, 20 µL overnight grown *C. sakazakii* isolates (10^6 cfu/mL) and extracts were added in the dose-dependent manner (50, 100 and 150 ppm) into 230 µL of fresh TSB medium. The plates were incubated without agitation for 48 h at 37 ^{o}C. After incubation, the ability of the extract to disrupt biofilm was assayed using CV staining as discussed in section. The percentage of biofilm inhibition was calculated by following the formula: percentage of biofilm inhibition = (control $_{OD570\ nm}$ − test $_{OD570\ nm}$ / control $_{OD570\ nm}$) × 100. The dose of 100 ppm illustrating strong anti-biofilm activity was taken as minimum inhibitory dose and used for further experiments.

Microscopic observation of biofilm

Light microscopic analysis

The light microscopy analysis of bacterial biofilm was carried out following the method of Musthafa et al. (2010). A 50 µL overnight grown *C. sakazakii* isolates (10^6 cfu/mL) were added into 2 mL of fresh TSB medium containing cover slips 1 x 1 cm in 24-well MTP along with a control well without extracts. After static incubation for 48 hours at 37 ^{o}C, the cover slips were removed, rinsed with phosphate buffer (pH-6.5) and were stained with 0.2% CV. The stained cover slips with the biofilm were visualized under light microscopy at magnification of 40X (Labomed CxL Monocular).

Scanning Electron Microscopy (SEM)

The biofilms of *C. sakazakii* isolates were obtained on glass cover slips as described earlier. After 48 h, the dehydrated biofilms were coated with the thin layer of gold and examined under SEM (Hitachi S-3400N, Tokyo, Japan) using an accelerating voltage of 10kV.

Fluorescent Microscopy

The biofilms on the cover slips were also visualized using the LIVE/DEAD *Bac* Light bacterial viability kit (Invitrogen-Molecular Probes) to stain cells over a 15 min period in the dark as per manufacturer's instructions. The bacteria were observed at 400X magnification using a fluorescence microscope (Olympus microscope, USA).

Statistical Analysis

The mean values (n=3) were calculated for biofilm formation and percent biofilm inhibition and the comparison between the means was done by ANOVA and Tukey's multiple comparison test ($P < 0.05$) by SPSS software.

RESULTS AND DISCUSSION

Objective 1: Isolation and characterization of *C. sakazakii* from different sources and detection of selected virulence traits in the *C. sakazakii.*

The present study reported the isolation and presence of *Cronobacter* spp. from a diverse variety of food and environmental samples in an attempt to pinpoint their reservoir (Table 2). From 219 samples, among 85 presumptive isolates on TSA, only 58 isolates resulted in blue-green coloration on chromogenic ESA. The biochemical tests carried out in the study revealed that all the 58 isolates as well as the reference strain ATCC 12

868 and E604 were positive for citrate utilisation. All *Cronobacter* spp. isolates were negative for the production of Indole and Methyl red. However, two isolates out of 58 isolates were negative for VP test. Further, the molecular characterization targeting 16S rRNA region resulted in 408 bp amplified product in the 45 isolates in addition to standard strains. The other 13 isolates did not give the expected PCR product although they were identified as *Cronobacter* spp. by chromogenic assay which imposes the relevance of more than one technique in precise identification of the *Cronobacter* spp. The species-specific PCR based on the ITS-G sequences and rpoB analysis confirmed 38 numbers of isolates as *C. sakazakii* (Table 2).

Table 2: Characterization of *C. sakazakii* from different sources using biochemical testing and PCR analysis

Isolate		IMVIC Test				PCR Primers			
ID	**Source**	**Indole**	**Methyl red**	**Vogus Proskauer**	**Citrate utilisation**	**16S rRNA**	**ITS-G**	**rpoB**	***fus*A**
E604	Standard strain	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*	*C. sakazakii*	*C. sakazakii*
ATCC 12868	Standard strain	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*	*C. sakazakii*	*C. sakazakii*
N1	Raw milk	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*	*C. sakazakii*	-
N2	Vegetable	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*	*C. sakazakii*	-
N3	Vegetable	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*	*C. sakazakii*	-
N4	Raw milk	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*	*C. sakazakii*	-
N5	Milk powder	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*	*C. sakazakii*	-
N6	Stool sample	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*	*C. sakazakii*	-
N7	*Coriandrium sativum*	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*	*C. sakazakii*	-
N8	*Syzygium aromaticum*	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*	*C. sakazakii*	-
N9	Milk powder	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*	*C. sakazakii*	-
N10	*Cuminum cymirium*	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*	*C. sakazakii*	-
N11	Stool sample	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*	*C. sakazakii*	-
N12	Fruit	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*	*C. sakazakii*	-
N13	Fruit	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*	*C. sakazakii*	*C. sakazakii*
N14	Turmeric powder	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*	*C. sakazakii*	*C. sakazakii*
N15	Soil	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*	*C. sakazakii*	*C. sakazakii*
N19	Legumes	-	-	+	+	-	-	-	-
N24	Market Snacks	-	-	+	+	*Cronobacter spp.*	-	-	-
N27	Biscuits	-	-	+	+	-	-	-	-
N31	Citrus fruit	-	-	+	+	*Cronobacter spp.*	-	-	-
N35	Baked bread	-	-	+	+	-	-	-	-
N41	Pasteurized milk	-	-	+	+	-	-	-	-
N46	Coriander	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*	*C. sakazakii*	-
N48	Gram flour	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*	*C. sakazakii*	-
N50	Black pepper	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*	*C. sakazakii*	-
N51	Large cardamom	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*	*C. sakazakii*	-

N52	Nutmeg	-	-	+	+	-	-		-
N53	Cumin	-	-	+	+	-	-		-
N54	Clove	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*	*C. sakazakii*	-
N56	Mango powder	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*	*C. sakazakii*	-
N57	Ginger	-	-	+	+	-	-		-
N61	Soil	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*	*C. sakazakii*	-
N63	Fenugreek	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*	*C. sakazakii*	-
N64	Cardamom	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*	*C. sakazakii*	-
N65	Fenugreek	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*	*C. sakazakii*	-
N69	Market Soup powder	-	-	+	+	-	-		-
N74	Vacuum Dust	-	-	-	+	*Cronobacter spp.*	*C. sakazakii*	*C. sakazakii*	-
N75	Soil	-	-	-	+	*Cronobacter spp.*	*C. sakazakii*	*C. sakazakii*	-
N77	Chilli powder	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*	*C. sakazakii*	-
N79	Cumin	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*	*C. sakazakii*	-
N81	Pond water	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*	*C. sakazakii*	*C. sakazakii*
N83	Sugar	-	-	+	+	*Cronobacter spp.*	-	-	-
N85	Rain water	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*	*C. sakazakii*	-
N87	Vegetable	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*	*C. sakazakii*	-
N88	Fruit	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*	*C. sakazakii*	-
N92	Chilli powder	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*	*C. sakazakii*	-
N96	Vegetable	-	-	+	+	-	-	-	-
N101	Flattened rice	-	-	+	+	-	-	-	-
N106	Fruit	-	-	+	+	*Cronobacter spp.*	-	-	-
N108	Coriander	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*	*C. sakazakii*	
N112	Fruit	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*	*C. sakazakii*	*C. sakazakii*
N118	Fruit	-	-	+	+	-	-	-	-
N119	Water	-	-	+	+	-	-	-	-
N121	Biscuit	-	-	+	+	*Cronobacter spp.*	-	-	-
N134	Chocolate	-	-	+	+	*Cronobacter spp.*	-	-	-
N148	Chips	-	-	+	+	-	-	-	-
N156	Fruit	-	-	+	+	*Cronobacter spp.*	-	-	-
N162	Salt	-	-	+	+	*Cronobacter spp.*	-	-	-
N174	Fruit	-	-	+	+	*Cronobacter spp.*	-	-	-

Owing to the ubiquitous nature of the organism and the high severity of infection, a rpoB anlaysis has been developed for the rapid and reliable identification and discrimination between different *Cronobacter* spp. The confirmed *C. sakazakii* strains and standard *C. sakazakii* strains were investigated for the further study.

The investigation of virulence factors and their interactions with the host is essential to understand the pathogenesis of any bacterial system. To detect the virulence marker genes, almost all the established virulence associated genes were screened in the present investigation to obtain comprehensive data on presence of these genes among *C. sakazakii* isolates of Indian origin. Our results revealed that among the tested isolates, all the isolates were capable to utilise inositol however, sialic acid utilization and DNase activity was observed in only two isolates, respectively. The presence of genes for inositol fermentation has been detected in two other meningitic strains of *C. sakazakii* (strains 701, 767) belonging to ST4 therefore recommended as a marker of pathogenicity for *C. sakazakii*. Sialic acid is present in breast milk, infant formula, the mucin lining of the intestinal tract and is also a constituent of the brain ganglioside complex. Among the different species of *Cronobacter*, *C. sakazakii* is the only species that has the *nanAKT* gene cluster encoding the utilization of sialic acid as a carbon source which aids in pathogenicity for neonates and young infants.

The importance of hemolysins in virulence of bacterial pathogens has been extensively characterized. Reports about the production of hemolytic toxins by C. *sakazakii* are very scarce. All the isolates resulted in haemolytic zone on blood agar plates. The other two determinants siderophore production was observed in single out of three isolates whereas chitinase activity was observed in all the isolates.

The carriage of nine virulence associated genes coding for *ompA, hly, cpa, chiF, eitA, zpx, sip, fhaB* and *iucC* was studied on genomic as well as plasmid DNA in *C. sakazakii* isolates (Table 3). All the isolates were having pESA2 and pESA3 plasmids. The ability of the opportunistic pathogen *Cronobacter* spp. to cause meningitis is dependent upon its capacity to survive in blood. All the *C. sakazakii* strains in this research were confirmed positive for *hly* gene, with one isolate observed negative for *ompA*. Kim et al. also reported that *ompX* and *ompA* was also crucial for *C. sakazakii* invasion of host cells (2010). Mittal et al. reported that o*mpA*$^{+}$ *C. sakazakii* isolates breach blood-brain barrier and invade central nervous system (CNS) therefore it is imperative for the commencement of meningitis in a neonatal mice model (2009). The type III hemolysin, an integral outer membrane protein with hemolytic activity is reported to a potential virulence factor in several pathogens. Earlier,

Cruz et al. (2011) reported the prevalence of *hly* gene in only *Cronobacter* spp. isolates mainly from clinical sources.

The capacity of iron acquisition is mostly believed to be a requirement for a pathogen to establish infections when entering a host (Crosa et al. 2002). Franco et al. (2011) demonstrated that plasmids pESA3 and pCTU1 in *C. sakazakii* contain two clusters of genes, a homologue of an ABC transport-mediated iron uptake and siderophore system a (*eitCBAD* operon) and a siderophore-mediated iron acquisition system (*iucABCD/iutA* operon). The gene *eitA* was frequently present in the isolates as well as in standard strains. It was observed that all the *C. sakazakii* strains lack genes namely *sip*, *fhaB* and *iucC*, however *iuc*C was present in both the standard *C. sakazakii* strains.

Chitinase is a putative virulence factor; and its expression has proven to be vital not only for nutrient acquisition and environmental survival but also for infection in humans and animals (Yan et al. 2011). The *chiF* gene and BAM were least commonly detected in the isolates. Besides, *zpx* gene encoding the cell-bound zinc-containing metalloprotease might to play a important role in by enhancing the dissemination of the pathogen into the systemic circulation (Kothary et al 2007). The least presence of *zpx* in our isolates indicates that there must be some other mechanisms in *Enterobacteriaceae* family which might play role in system.

Table 3: Virulence traits identified in *Cronobacter sakazakii* isolates

Strains	Plasmid		Virulence determinants through biochemical assays						Detection of virulence associated genes by PCR						
	pESA3	pESA2	Inositol Fermentation	DNase activity	Hemolytic activity	Siderphore detection	Chitinase activity	Sialic acid utilization	*omp*	*hly*	*cpa*	*chi*F	*eit*A	BAM (*zpx*)	*iuc*C
E604	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
ATCC 12868	+	-	+	+	+	+	+	+	+	+	+	-	+	+	+
N13	+	+	+	+	+	-	+	+	+	+	+	-	-	-	-
N14	+	+	+	-	+	-	+	-	+	+	-	-	+	+	-
N15	+	+	+	-	+	-	+	-	-	+	+	-	+	-	-
N81	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-
N112	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-

+ indicates the presence of respective trait; - indicates the absence of respective trait

Objective 2: Characterization of biofilm in *C. sakazakii* and determination of key molecules involved in quorum sensing.

Quantification of Biofilm Formation

The formation of biofilms by pathogens on biotic and abiotic surfaces increases the risk of infections by increasing their resistance or tolerance to environmental stresses and resistance to disinfectants and other commonly used antimicrobial compounds. For the quantification of the biofilm, a SBF index was calculated for each strain which represents the most convenient mathematical formula to quantify the expression of bacterial biofilms. A strong SBF index was observed for all the tested strains at the interface of polystyrene microtiter well (p <0.05) (Table 4). The strong index indicates that the strains significantly formed a biofilm under prolonged periods of incubation (48 h). Strain E604 demonstrated a strong SBF as compared to other strains tested. Lehner et al. (2005) did not find any significant difference in biofilm forming abilities between the *C. sakazakii* isolates from human, environmental and foods whereas, Lee et al. (2012) reported significant variation in biofilm formation among different food isolates of *Cronobacter* spp. The variation in the biofilm forming abilities is reported to be strain-specific as reported in other studies in *C. sakazakii*. The presence of cellulose biosynthesis genes (*bcsC, bcsA* and *bcsG*) was further analyzed among the *C. sakazakii* strains (Table 4). All the tested strains possessed gene encoding for peptidoglycan hydrolase (*flgJ*) whereas the genes encoding for flagellar synthesis had different prevalences among the strains. Our results revealed that the genes for biosynthesis of cellulose are present in tested C. *sakazakii* strains which also possessed a higher SBF index value. The study by Hu et al. (2015) also reported the prevalence/presence of *bcsA* (93.3%), *bcsB* (95%) and *bcsC* (100%) in 180 *C. sakazakii* isolates and also in *C. sakazakii* BAA-894 indicating that *bcsABC* genes are necessary to produce cellulose, and are involved in biofilm formation. Our results also indicated the presence of *flgJ* in all the strains with the presence of *fliD* and *flhE* varying among the strains. These findings signified that the *bcsABC* genes are some of the genes present that may direct or regulate the biofilm formation. However, pleiotropic mechanisms still need to be understood since biofilm formation consists of series of complex processes which might be commonly regulated by the interaction between different genes and various environmental conditions.

EPS characterization

Since the composition of the EPS can influence biofilm architecture, we investigated the total carbohydrates and concentration of proteins of the strains. The composition of EPS expressed by *C. sakazakii* possessed very low protein:carbohydrate content and EPS expression differed

among the isolates ($p < 0.05$) (Table 4). The FTIR spectroscopy of the EPS revealed the presence of COOH groups (1600 cm^{-1} to 1725 cm^{-1}) and -OH (2800 cm^{-1} to 3600 cm^{-1}) groups, indicating that the samples were exopolysaccharide. The presence of -CH- vibrations in lipids, amide I in proteins, amide II in protein and -COC- group vibration in carbohydrates was indicated by the peaks obtained at wave no. range 3,200-2,800, 1,800-1,600 and 1,600-1,500, respectively.

AHL screening and characterization

It is well known that the biofilm-forming ability of bacteria is mainly due to production of AHL mediated QS. In our study, we did not observe any QS signal molecule using the CV026 biosensor indicating the low production levels of short acyl AHLs (C4-AHL to C8-AHL). These short acyl AHLs can only be detected at higher concentrations as earlier reported in *P. aeruginosa* which either failed to produce short chain AHLs or the level of signals was very low. However, the production of blue-green chromogenic reaction in the *A. tumefaciens* NTL4(pZLR4) and X-gal indicated the presence of long chain AHLs as signalling molecules in *C. sakazakii* (Fig. 2). Further LC-HRMS analysis confirmed the expression of long chain AHLs among the tested strains. Taken the biosensor assay and mass spectra data together, it is unequivocal confirming the presence of long chain AHL in the *C. sakazakii.*

Based on the SBF index, isolate N13, N15 and N112 were selected for further work. All the isolates produced colourless colonies with the CV026 bioassay and produced blue green color colonies with the *A. tumefaciens* NTL4(pZLR4) bioassay indicating that the strains were expressing long chain AHL signalling molecules.

The FTIR analysis of the AHL showed the presence of a lactone ring, N-H bond, C-O bond by peak at 1664.33 cm^{-1}, 1428.99 $cm^{-1}$and 1179.90 cm^{-1}, respectively. In HPLC analysis, the chromatogram of 3-oxo-C8 AHL (standard) two peaks were observed at 8.92 min and 85.11 min, indicating elution of solvent (DMSO) and 3-oxo-C8, respectively. A single peak was observed in a test sample at 84.74 min showed the presence of AHL. The LC-HRMS analysis of AHL confirmed the presence of N-undecanoyl-L-AHL, N-dodecanoyl-L-AHL, N-tetradecanoyl-L-AHL, N-pentadecanoyl-L-AHL, N-(β-ketocaproyl)-L-AHL, N-octanoyl-L-AHL, N-3-oxo-octanoyl-L-AHL and N-octadecanoyl-L-AHL (Fig. 1).

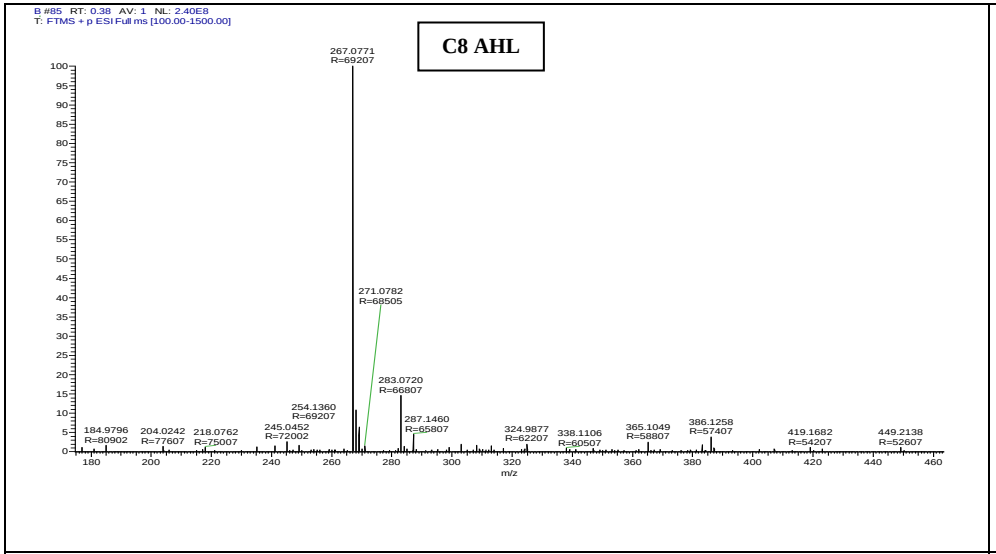
Table 4: Biofilm formation, composition and prevalence of biofilm forming genes among *C. sakazakii* isolates

C. sakazakii strains	Biofilm Formation and Quantification			EPS Composition		Genes involved in biofilm formation					
	BF=AB-CW	**BF=AB/CW**	**SBF=(AB-CW)/G**	**Total Carbohydrate (µg/ml)**	**Total Protein (µg/ml)**	***bcsC***	***bcsG***	***bcsA***	***flgJ***	***fliD***	***flhE***
ATCC 12868	3.2	8.06	2.95^{f}	79.21^{d}	0.58^{c}	+	+	+	+	+	+
E604	3.7	9.32	4.73^{g}	87.06^{f}	7.11^{g}	+	+	+	+	+	-
N13	1.87	4.71	1.90^{c}	55.31^{c}	5.06^{f}	+	+	-	+	-	+
N14	1.21	3.05	1.02^{a}	45.51^{b}	0.10^{b}	-	+	+	-	-	-
N15	2.98	7.51	2.27^{d}	82.76^{e}	4.70^{e}	+	-	+	+	+	-
N81	1.35	3.40	1.39^{b}	33.6^{a}	0.08^{a}	-	+	-	+	-	-
N112	2.95	7.43	2.48^{e}	121.67^{g}	1.93^{d}	+	+	+	+	-	-

+ indicates the presence of respective trait; - indicates the absence of respective trait

a-g = Different letters in the same column indicate that the values are significantly different ($p < 0.05$) as measured by 2 sided Tukey's – post-hoc range test between replications

Previously, Lehner et al.(2005) tentatively indicated the presence of two different types of AHLs (3-oxo-C6-AHL and 3-oxo-C8-AHL) by Thin Layer Chromatography (TLC) which predicted the ability of *Cronobacter* spp. to produce cell-to-cell signalling molecules. Still, there are no other reports on the expression of QS molecules and their role in biofilm formation in C. *sakazakii*. Our results reflect that the isolates which have significant levels of long chain AHLs expression also showed higher SBF indices. These results are in accordance with the findings of Taghadosi et al. (2015) who reported that *E. coli* isolates with the highest AHL levels also exhibited strong adherence to microplate wells. Khajanchi et al. (2009) proposed that AHL-mediated QS signalling system modulates the virulence of *Aeromonas hydrophila* by regulating the T6SS, metalloprotease production and biofilm formation.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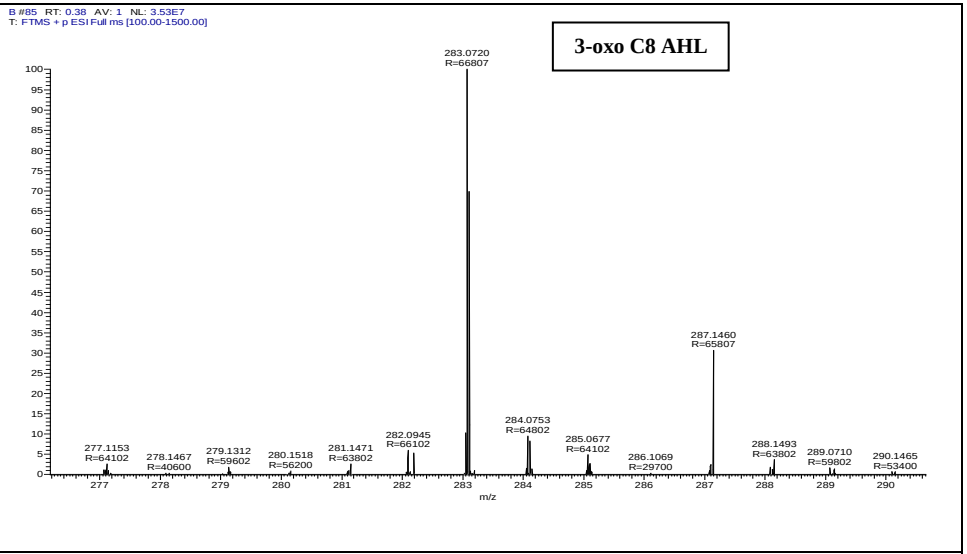
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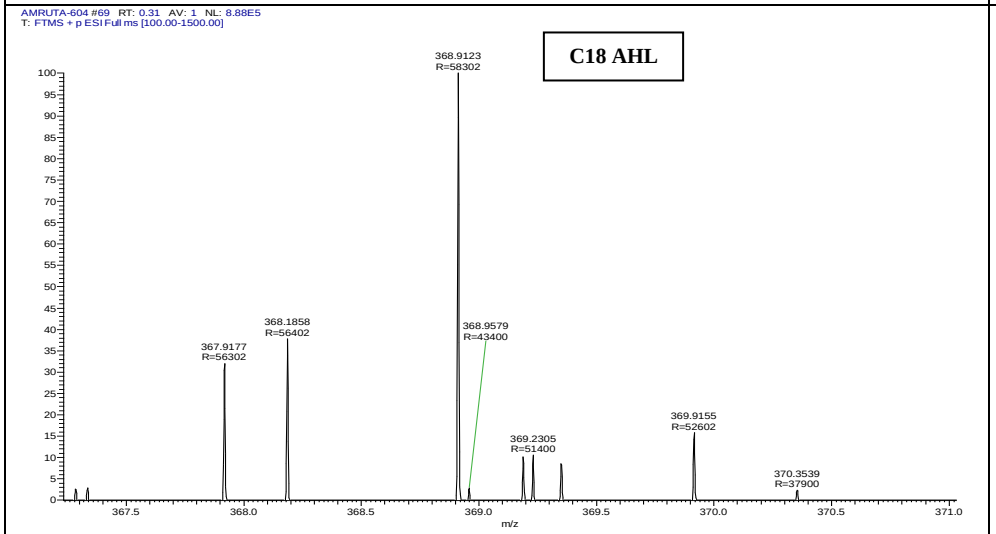
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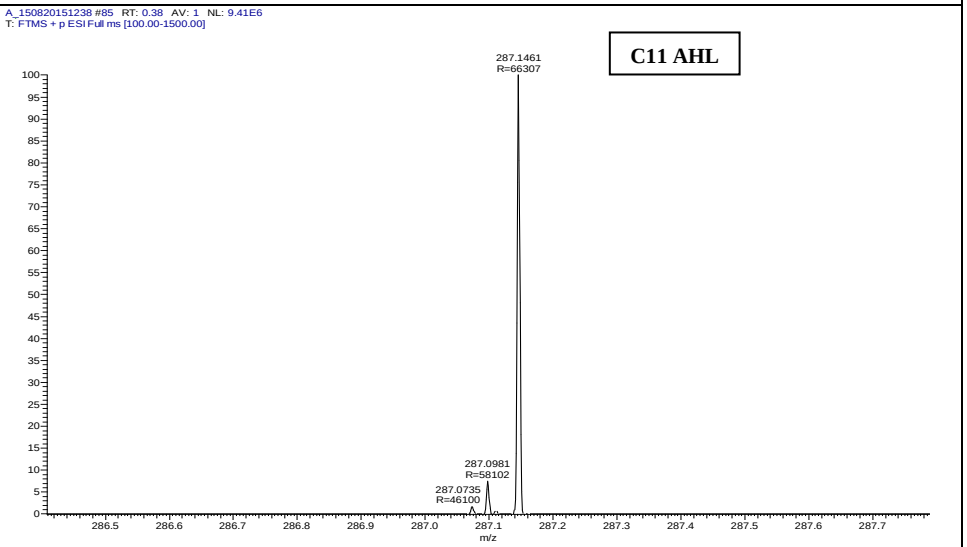
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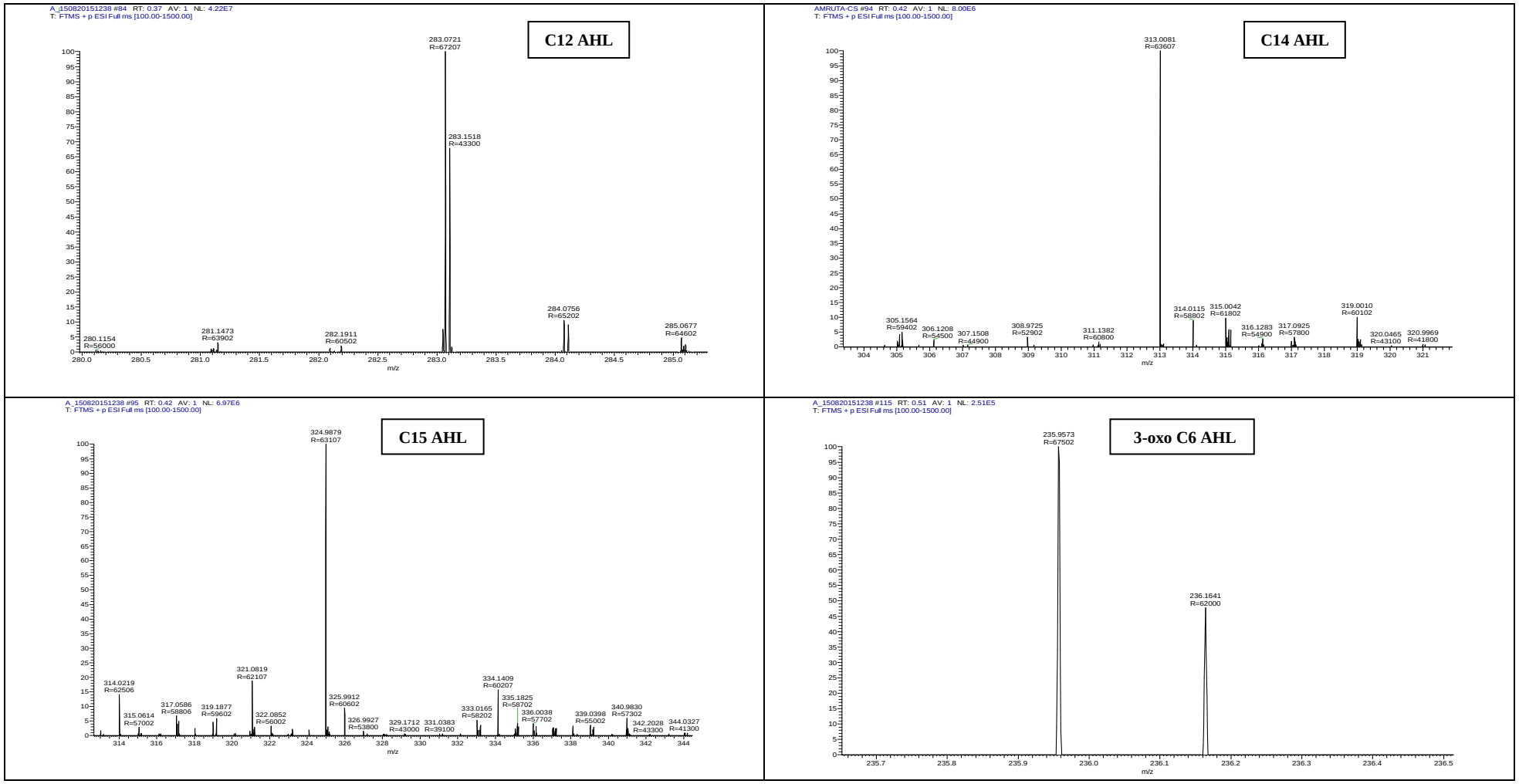

Fig. 1: LC-HRMS profiles of AHL signals: Detection of AHLs by mass spectrometry analysis of supernatants extracts of *C. sakazakii*. A. C8 AHL (Retention time: 22.8 sec; m/z: 267.07) B. 3-oxo-C8 AHL (Retention time: 22.8 sec; m/z: 283.07) C. C18 ASL (Retention time: 18.6 sec; m/z: 368.91) D. C11 AHL (Retention time: 22.8 sec; m/z: 287.14) E. C12 AHL (Retention time: 22.2 sec; m/z: 283.07) F. C14 ASL (Retention time: 25.2 sec; m/z: 313.008) G. C15 AHL (Retention time: 25.2; m/z: 324.98) H. 3-oxo-C6 AHL (Retention time: 30.6 sec; m/z: 235.95)

Objective 3: Evaluation of quorum sensing inhibitory activity of plant extracts against biofilm forming *C. sakazakii.*

Inhibition of quorum sensing by plant extracts

For anti-quorum sensing activity, methanolic extracts of nine plant products (*Piper nigrum, Trigonella foenum-graecum, Coriandrum sativum, Cuminum cyminum, Syzygium aromaticum, Myristica fragrans, Zingiber officinale, Allium sativum and Cinnamomum verum*) were tested for their potential to inhibit biofilm formation in *C. sakazakii* strains.

Minimum Inhibitory Concentration (MIC) was determined for the plant extract (50-300 ppm) against *C. sakazakii* strains. The test extract showed growth inhibition against all the test strains beyond 150 ppm, the MIC was found to be 200 ppm for garlic and clove extract and 250 ppm for rest of the extracts. Hence, in the present study, sub-MIC concentrations (50-150 ppm) of test extract were used for further experiments.

The qualitative analysis of the extracts (100 ppm) against biosensor strain exhibited QS inhibitory activity. The loss of blue-green color in *A. tumefaciens* NTL4(pZLR4) (with added AHL) was detected mainly in *Cin. verum* and *Cum. cyminum* with a zone of pigment inhibition ranging from 10-12 mm. Interestingly, the extracts showed a pigmentless zone with both the biosensor strain around the wells. The lawn of bacteria in this zone indicated living cells with loss in their QS ability. These results demonstrated that extracts might have obstructed the interaction between the added AHL (e.g., 3-oxo-C8 AHL) and their receptors (e.g. TraR for *A. tumefaciens* NTL4(pZLR4). However, the other plant extracts did not show anti-QS activity. No inhibition was observed with 10% DMSO used as control. Niu *et al*. (2006) reported that cinnamaldehyde (compound of *Cin. verum*) is a potential inhibitor of 3-oxo C6 AHL in the biosensor strain *E. coli* and was also found to inhibit QS in *Vibrio harveyi* by inhibiting the 3-hydroxy C4 AHL QS signaling molecule at sub-inhibitory concentrations. The quantitative assay showed significant loss (68%) of blue-green color was detected in *A. tumefaciens* NTL4(pZLR4) with extracts from *Cin. verum* while *Cum. cyminum* and *P. nigrum* resulted in a reduction of 21.5 and 16.2%, respectively (Fig. 2). Negligible effect on inhibition was noted by other plant extracts.

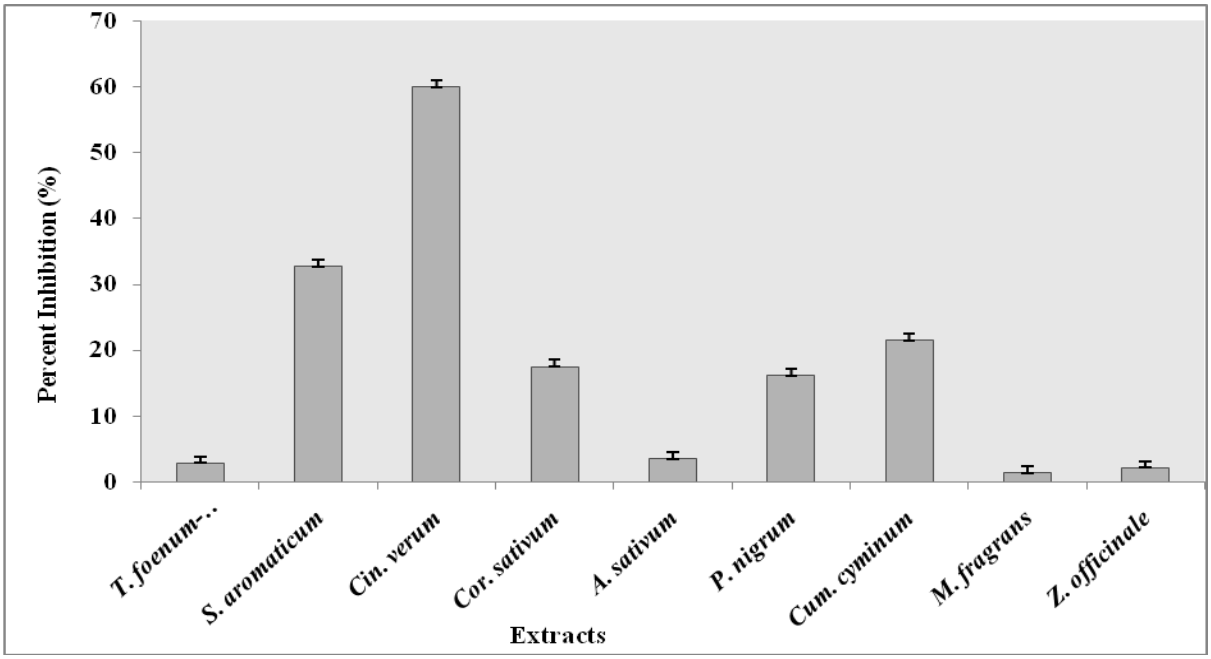

Fig. 2: Quantitative analysis of inhibition of blue-green color in *A. tumefaciens* NTL4(pZLR4) by plant extracts.

Inhibition of *C. sakazakii* biofilm formation

The formation of biofilm plays an important role in the pathogenesis of *C. sakazakii* and developments of these biofilms are based on the signal-mediated QS system. Therefore, an interference with QS may prevent the development of bacterial biofilms and further infections. Confirmation of the biofilm-inhibitory activity of the nine plant extracts was determined at different doses of plant extracts. A significant biofilm inhibition was observed with the plant extract as compared to the negative control, when the extracts were used at a dose of 100 ppm ($P < 0.05$). The extracts were divided into three groups depending on percent biofilm inhibition (high, > 50%; moderate, 50-25% and minimal, < 25%) against *C. sakazakii* isolates. Four (*P. nigrum, S. aromaticum, T. foenum-graecum and Cin. verum*) of the nine extracts investigated, possessed higher anti-biofilm activity against the strains (Table 5). While the other extracts showed moderate or minimal inhibitory effect on the pathogens. The *P. nigrum* and *Cin. verum* showed a maximum reduction in biofilm biomass ranging from 55-75% against all the tested strains. Moderate inhibitory activity was exhibited by *Cor. sativum* (36-53 %) and *A. sativum* (23-56 %) while *Cum. cyminum, M. fragrans* and *Z. officinale* showed minimal activity against biofilm forming *C. sakazakii* isolates.

The results of light and SEM microscopic images revealed clear differences in biofilm structure between biofilms treated with *P. nigrum* extract and the untreated control (Fig. 3).

The control slides showed a well developed biofilm growth of the test strain, whereas, on treatment with plant extract, scattered cell growth was observed on the glass slide. Further, fluorescent microscope images indicated well developed biofilm in control whereas the strains were treated with extract developed poor biofilm. (Fig. 4). These observations clearly reveal the ability of *P. nigrum* extract to disturb the mature biofilms.

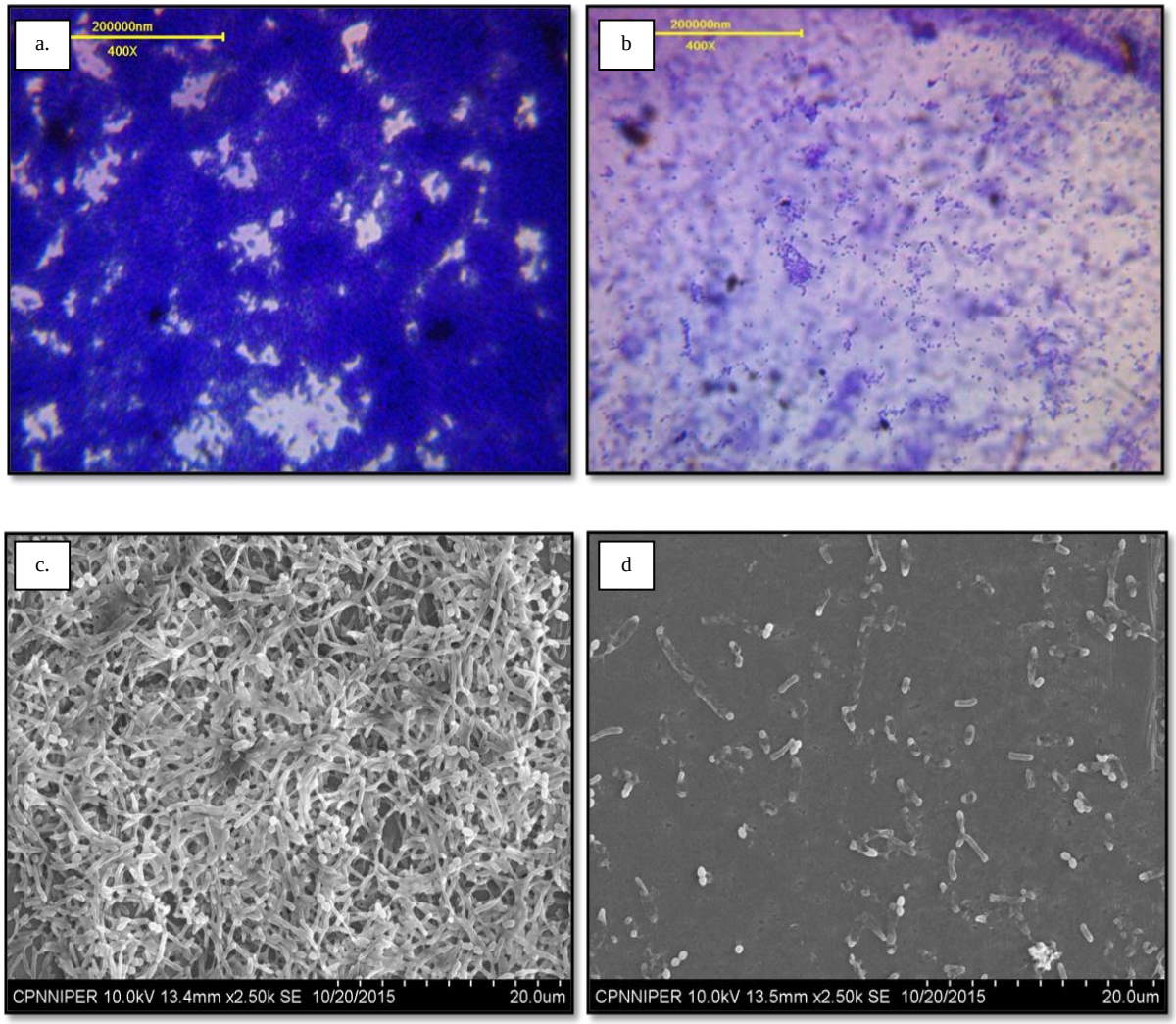

Fig. 3: Light and Scanning electron microscope images of biofilms of *C. sakazakii* isolate grown in the absence (a, c) and presence of methanolic extract of *P. nigrum* (100 ppm) (b, d), respectively

Table 5: Effect of plant extracts on inhibition of biofilms (%) for *C. sakazakii* strains.

Test strains	Total Biofilm (OD 570nm)	Biofilm inhibition (%)								
		T. foenum-graecum	*S. aromaticum*	*Cin. verum*	*Cor. sativum*	*A. sativum*	*P. nigrum*	*Cum. cyminum*	*M. fragrans*	*Z. officinale*
E604	1.79	47.16^{e}	25.47^{b}	73.85^{h}	48.83^{e}	56.61^{f}	68.85^{g}	31.6^{c}	36.54^{d}	11.67^{a}
ATCC 12868	1.65	35.15^{a}	55.09^{de}	56.36^{e}	50.9^{cd}	56.36^{e}	72.12^{f}	40.6^{b}	49.81^{c}	44.84^{b}
N13	1.11	58.34^{f}	23.24^{c}	41.65^{e}	36.17^{d}	23.69^{c}	65.88^{g}	27.2^{c}	2.87^{a}	14.18^{b}
N15	2.33	48.67^{b}	53.42^{c}	59.79^{d}	54.4^{c}	47.39^{b}	60.65^{d}	39.26^{a}	41.57^{a}	40.5^{a}
N112	1.76	59.38^{d}	65.34^{ef}	69.93^{f}	53.03^{c}	45.54^{b}	61.99^{de}	47.36^{b}	43.44^{b}	38.06^{a}

Data bearing same superscript letters are not significantly different ($p < 0.05$) among tested methnolic plant extracts as determined by 2 sided Tukey's – post-hoc range test between replication.

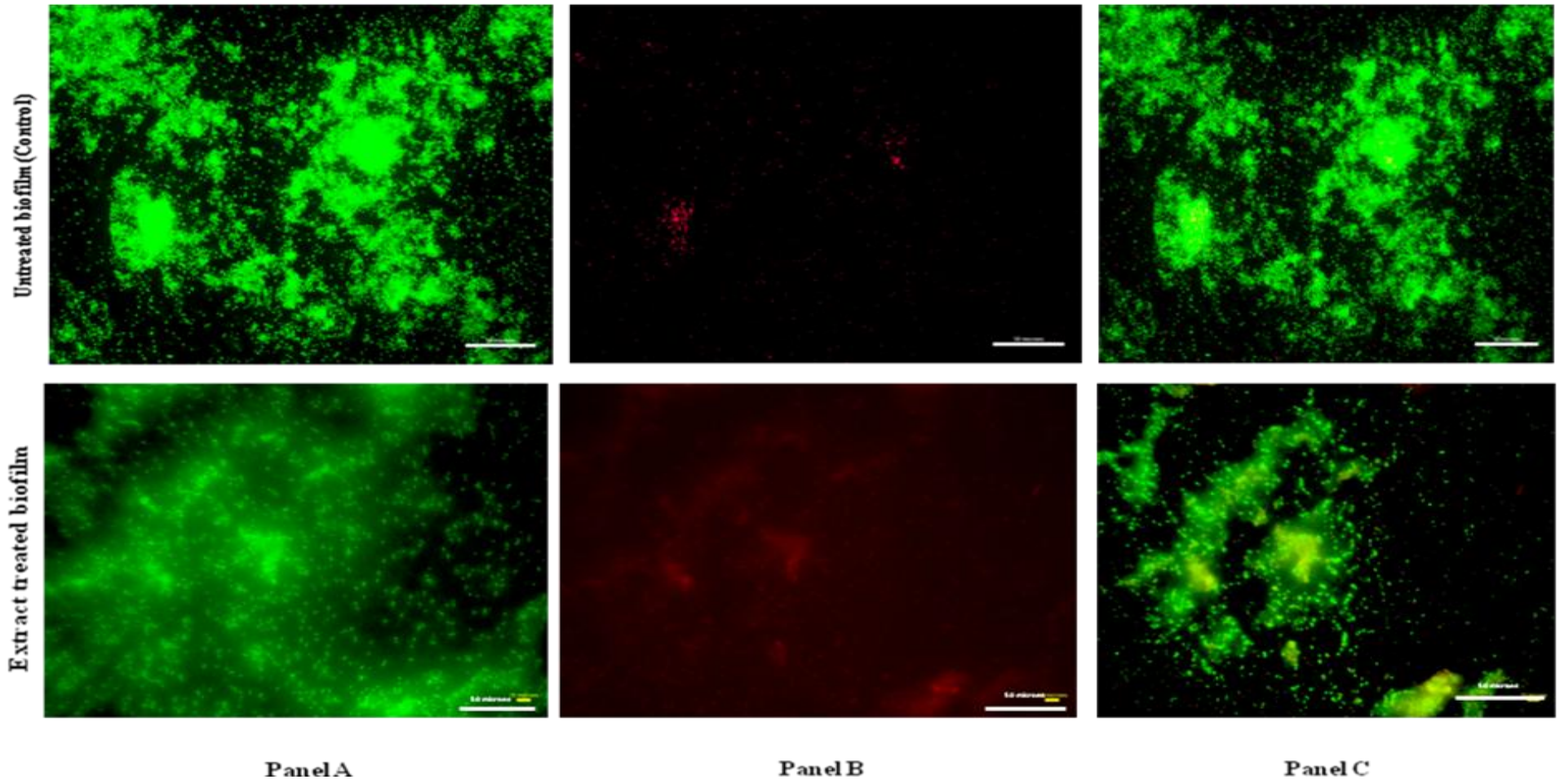

Fig. 4: Fluorescent microscopic images of biofilms of *C. sakazakii* grown in the absence and presence of methanolic extract of *P. nigrum* (100 ppm). Panel A (left) is the image obtained from the green channel, panel B (center) from the red channel and panel C (right) is a merged image.

CONCLUSION AND FUTURE PROSPECTS

The present study showed that *C. sakazakii* are highly diverse and share many phenotypic traits with other *Enterobacteriaceae* members highlighting the need to use several methods to confirm the identity of this pathogen. Our results revealed information on frequency of virulence gene i.e., the very high presence of *hly* followed by *ompA* and *eitA* gene which elucidates the role in the pathogenesis of enteric infections and in its systemic dissemination. A larger sample size of each source would be necessary to further address differences observed. Further, this is the first report describing the expression of long chain AHLs in *C. sakazakii* and confirmed the presence of 3-oxo-C8-AHL. Since QS regulates the expression of many important phenotypes including virulence production, therefore the study of QS in *C. sakazakii* may lead to better understanding its survival in the different environment and host. The tested plant extracts reported in the current investigation efficiently inhibited AHL based QS mechanisms in *C. sakazakii* along with their ability to disrupt the biofilms of the pathogen. In the interest of food safety, this study introduces the QSI and anti-biofilm potential of plant extracts which can be easily incorporated as food ingredient to limit the biofilm forming ability in *C. sakazakii* from different processing environments. Further work should focus on chemical identification of the anti-quorum sensing compounds using chromatographic methods and mass spectrometry. The purified compounds could be used for inhibition of QS signaling system, which could offer an opportunity to develop new drugs to combat pathogens. The findings reported in the present study are likely to boost the morale of the scientific community to develop effective strategies for the eradication of biofilm formation by *C. sakazakii*

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List of Publications

- **Singh N**, Goel G and Raghav M (2015). An insight into putative virulence factors determining the pathogenicity of *Cronobacter sakazakii*. Virulence 433-440. (IF-5.418)
- **Singh N**, Goel G and Raghav M (2015). Prevalence and characterization of *Cronobacter* spp. from various foods, medicinal plants and environmental samples. Current Microbiology 71:31-38.(IF-1.519)
- **Singh N**, Patil A, Prabhune A, Raghav M and Goel G (2016). Diverse profiles of N-acyl-homoserine lactones and biofilm formation in *Cronobacter sakazakii.* Virulence (In press). (IF-5.418)
- **Singh N**, Patil A. Prabhune A and Goel G (2016). Inhibition of quorum sensing mediated biofilm formation in *Cronobacter sakazakii* strains. Microbiology (In press) (IF-2.2).
- **Singh N**, Raghav M, Narula S, Tandon S and Goel G. (2016). Prevalence and profiling of virulence determinants in *Cronobacter sakazakii* isolates from different food and environmental commodities. Current Microbiology (Minor revision). (IF-1.519)

Book Chapter:

- **Niharika Singh,** Sampan Attri, Kavita Sharma and Gunjan Goel (2016). Indigenous Fermented Foods and Beverages. In Frontiers in Food Biotechnology. NOVA Publisher, pp 47-74 [ISBN: 978-1-63484-679-0].

Conference Publications

- **Singh N,** Patil A, Prabhune A, Goel G. Detection and characterization of Quorum Sensing signal molecules of *Cronobacter sakazakii*. International Conference on Frontiers of Mass Spectrophotometry (ICMS-2015), 11-14th Dec. 2015, Kottayam, Kerala, India.
- **Singh N**, Raghav and Goel G. Plasmid encoded virulence traits in *Cronobacter sakazakii*. The 6th Congress of European Microbiologists (FEMS 2015 Congress), 7-11th June, 2015 Maastricht, The Netherlands.
- **Singh N**., Raghav M. and Goel G. Phenotypic and genotypic characterization of environmental and food isolates of *Cronobacter* spp. other than infant formula. International Conference on Emerging Trends in Biotechnology (ICETB 2014), JNU, 6-9th Nov. 2014.
- **Singh N.**, Goel G, Raghav M, Rajani CS and Puniya AK. Indian spices as modulators of quorum sensing in *Cronobacter sakazakii*, a food borne pathogen. EUROBIOFILMS Third European Congress on Microbial Biofilms, European Society of Clinical Microbiology and Infectious Diseases. Ghent, Belgium, 9-12th Sep 2013.

- **Singh N**. and Goel G. Tannase: A promising enzyme in value addition to food products. 1st International Conference on Innovations in Food Processing, Value Chain Management & Food Safety (IFpvs), NIFTEM, 10-11th Jan. 2013.